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13. ABSTRACT *(Maximum 200 Words)*

The antiestrogen, tamoxifen, is currently the most effective hormonal agent for the treatment of breast cancer. The presence of nuclear estrogen (ER) and progesterone (PR) receptors in tumors is a strong prognostic marker for tamoxifen responsiveness. However, most tumors that initially regress, eventually become tamoxifen "resistant" and resume growing. Resistant tumors usually retain their ER. Tamoxifen is a mixed agonist/antagonist, a property that it shares with the antiprogestin RU486. Under certain conditions, mixed antagonists can impart strong, agonist-like, transcriptional activity. We postulated that in tamoxifen-resistant breast cancers, tamoxifen acquires strong agonist-like properties which override its antagonist effects. As a result, tamoxifen acts like an estrogen and enhances tumor growth. Our hypothesis as to the mechanism of hormone resistance involves the inappropriate recruitment of coregulatory proteins to the transcriptional machinery that switches the affect of antagonist hormones on their respective receptors. To define the molecular events that cause such a switch we used an antagonist-driven yeast two-hybrid screening strategy and isolated three antagonist-specific, receptor-interacting peptides. Our long-term goal is to understand how tamoxifen resistance develops, with a view to either preventing or reversing it.

14. SUBJECT TERMS
Progesterone receptor, Estrogen Receptor, tamoxifen, RU486, breast cancer, hsp90, Endocrinology, hormone resistance, yeast two-hybrid, agonist, antagonist, coactivator, corepressor, chaperonin, heat shock proteins,, PR, ER

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Table of Contents

Introduction

Subject: Estradiol is a human sex hormone and a major regulator of breast cancer growth. For this reason the estradiol inhibitor (or antiestrogen) tamoxifen, is the major drug used to treat breast cancers whose growth is "hormone dependent". Tamoxifen therapy often leads to reduction in tumor size. Unfortunately, despite continuing treatment, most tumors eventually become "resistant" to tamoxifen and resume growing. How does resistance develop? We now know that in the nucleus of breast cancer cells, estradiol binds to a protein called the estrogen receptor (ER), which regulates genes involved in growth. Ordinarily, tamoxifen displaces estradiol on ER and inhibits growth. However, tamoxifen has dual actions -- sometimes instead of being inhibitory (or "antagonist") which is desirable, it becomes a growth stimulator (or "agonist"). This is highly undesirable, and dangerous to the patient. We propose that tamoxifen can switch between the agonist and antagonist states by recruiting proteins called corepressors or coactivators, to genes that control growth. Tamoxifen would be inhibitory in the presence of corepressors; it would be stimulatory in the presence of coactivators. This idea leads to the suggestion that the overall levels of corepressors vs. coactivators in a tumor dictate whether tamoxifen is inhibitory or stimulatory. We further hypothesize that in "resistant" tumors there is an excess of the coactivators.

Purpose: To define the molecular events that cause such a switch we used an antagonist-driven yeast two-hybrid screening strategy to try to isolate antagonist-specific, receptor-interacting peptides. These proteins, while present in the cell and having possibly other functional roles in growth and development, may be inappropriately recruited to the transcriptional complex by antagonist bound transcription factors. There are a few already described coactivator and corepressor proteins that specifically bind to antagonist-bound steroid receptors, namely L7/SPA and SMRT, N-CoR, respectively. We believe that these proteins only represent a small fraction of the regulatory proteins that influence the activities of antagonist-bound transcription factors.

Scope: In work described in our previous report, we had isolated two novel peptides and a third peptide that was identified as NIP7 (or Ccth, the eta subunit of a hetero-oligomeric chaperonin complex that assists in actin and tubulin folding). The two novel peptides were designated as ORF#93 and ORF#127. ORF#93 demonstrated a specific interaction to the C-terminus of the progesterone receptor when bound with RU486 but not with R5020 (a synthetic progesterone) or no ligand. Sequence translation and analysis of the ORF#93 cDNA produced a peptide that contains two nuclear receptor (NR) boxes with the motif of LXXLL (where L = leucine and X = any amino acid). The presence of this motif within the peptide was particularly intriguing because it was originally described as a critical and complete motif required for coregulatory protein interaction with steroid receptors. Results within this report are a continuation of the characterization of ORF#93 and its functional relevance in transcription factor activity.

Aim 1: To clone, sequence, and define the structure of three novel antagonist-specific, ER and PR-interacting proteins.

The cloning of the full length cDNA of ORF#93 isolated in the original antagonist biased two-hybrid screen was performed previously by a second library screen using primers to the original peptide fragment and 5' anchor primer to a λ-gt11 HeLa cell cDNA library. The original peptide fragment was 393 amino acids (aa) long with a STOP codon and polyA tail and the resulting overlapping fragment translated in frame with the original fragment and extended the protein to 923 aa in length. This second region of ORF#93 contained **two more NR boxes and three tetratricopeptide repeat (TPR) domains** but no defined transcriptional start site. Using 5'-RACE, the final 21 N-terminal amino acids were cloned and identified (**Fig. 1**). During the final stages of the ORF#93 cDNA cloning, the genomic sequence of chromosome 15 was found in the high throughput genomic database (HTGS, accession # AC004886) that contained the ORF#93 sequence. Using the cloned cDNA sequence and the genomic sequence as a map, we were able to determine the genomic structure of ORF#93 and identify exon/intron junctions. ORF#93 is found on **chromosome 15q26.1 and is divided into at least 19 exons (Fig. 2)**. The working draft of the sequencing project is not complete and the contigs published in genebank are unordered. There is a stretch of 472 bps within the cDNA of ORF#93 that has not been sequenced/published in the HTGS. This region is designated as the single exon 9, however, this region could be comprised of several exons with intervening introns.

Cloning of the ORF#61 (NIP7, Ccth) was not necessary as the full length cDNA had already been isolated and subcloned by Kwang-Ai Won in Dr. S. Reed's lab at the Scripps Research Institute, La Jolla, CA. Dr. Won kindly has provided the cDNA on an expression vector for our use in further testing against PR and ER. Testing of this protein and its functional relevance to PR and ER gene regulation and function are planned but not yet completed.

ORF#127 mapped to the q-arm of chromosome 22 and a BLAST search of the genebank database identified it as a SCO-1 like gene. Sco1p in yeast is an inner membrane protein required for the recruitment of cytochrome c oxidase subunits within the mitocrondria. A 140 Kb BAC clone of chromosome 22 was obtained from The Sanger Center that contains the ORF#127 sequence. Isolation and subcloning of the full length cDNA of this novel PR interactor has not yet been finished. Further studies with this protein are pending the successful cloning of the full length cDNA.

Aim 2: To define the trascriptional coregulatory or other functional properties of the three receptor-interacting proteins.

We chose to focus on ORF#93 and therefore all subsequent functional experiments involve this gene and its protein product. In collaboration with Dinny Graham, Ph.D., David Toft, Ph.D., and Ahmed Chaldi, Ph.D., we have performed many functional assays on ORF#93 in an attempt to elucidate its function. **Site-directed mutagenesis** of the two C-terminal nuclear receptor (NR) boxes in the initial progesterone receptor (PR) interacting domain was carried out to determine whether these motifs were responsible for the PR-ORF#93 interaction (**Fig. 3**). A substitution

mutation in the second leucine of the NR box motif (LXXLL) to an alanine residue within NR3 and NR4 demonstrated that **NR3 is the region of PR interaction** while the mutation at NR4 had no effect. The interaction between PR and ORF#93 was also antagonist dependent as the two-hybid screen did not demonstrate an interaction in the absence of RU486. The **ORF#93 transcript size** was confirmed by Northern blotting total RNA from HeLa and T47Dco cell lines with an ORF#93 specific probe (**Fig. 4**). The ORF#93 message is expressed as a single species band of 3.6 Kb in size.

Functional experiments in collaboration with Dr. Dinny Graham failed to convincingly show an effect of ORF#93 on the activity of either PR or ER (data not shown). However, other experiments continued to confirm the interaction of ORF#93 with PR-A and PR-B (data not shown). We therefore began to direct our attention to the N-terminal region of ORF#93 and the presence of three **tetratricopeptide repeat domains** as an indication of the protein function. Many proteins have been described with this motif and have been shown to bind chaperonin proteins like hsp90 (**Fig. 5**). We have entered into a collaboration with Dr. David Toft of the Mayo Clinic in Rochester, MN to determine if ORF#93 is part of heat shock protein complex and whether it is involved in the maturation and/or selective binding of hormones to activated PR. Dr. Toft along with Dr. Chaldi were able to show that **ORF#93 is recruited in PR reconstituted complexes with purified chaperones** (**Fig. 6**). Using a chick PR specific antibody resin, Dr. Chaldi was able to demonstrate that ORF#93 binds in the reconstituted complex that includes PR, hsp90, hsp70, Hop, hsp40, and p23.

A second chaperoning experiment was done to show that **ORF#93 interacts with PR-A and PR-B in a complex** with hsp60, hsp90, hsp40, hsp70, and p23 (**Fig. 7A**). The blot shows that ORF#93 interacts with the chaperonin complex in the presence of PR. Lane 4 also demonstrates that the ORF#93 interaction with the complex is mediated through its interaction with PR. Experiments that include ORF#93 and the chaperonin complex in the absence of PR are planned but not yet completed as of this writing. Using a radiolabeled progesterone ligand in a binding assay, Toft's group was able to show that the binding of **ORF#93 inhibits the binding of progesterone to PR** (**Fig. 7B**). While ORF#93 and uncomplexed PR do not bind progesterone (lanes 2-4), the chaperonin complex with PR binds progesterone (lane 5, arbitrarily set to 100%) and the presence of ORF#93 inhibits binding by 52% (lane 6).

Dr. Toft's group made a monoclonal antibody to ORF#93 and was able to show that the ORF#93 protein product is **expressed in the cytosol of rat brain** (**Fig. 8**) as well as **expressed in different ovarian cell lines** (**Fig. 9**). Thus this monoclonal antibody, unlike the polyclonal antibody that we produced (not shown), is capable of detecting the endogenous protein. Studies of the protein in breast cancer cells are underway.

Key research accomplishments:

- ✓ A full length cDNA clone of ORF#93 has been obtained and sequence confirmed which may be involved in the maturation and/or hormone binding specificity of PR.
- ✓ The genomic location of gene#93 has been determined to be on chromosome 15q26.1.
- ✓ The organization of gene#93 has been determined to consist of at least 19 exons with intervening introns of various sizes.
- ✓ Structural motifs within the translated protein have been identified that may indicate the function of the protein.
- ✓ Specific binding of the interaction domain of ORF#93 has been determined to be mediated through a nuclear receptor (NR) box domain (LXXLL motif).
- ✓ Binding of ORF#93p to the hinge-HBD C-terminus of PR through the NR box is antagonist hormone dependent.
- ✓ Gene #93 consists of an ORF of 2832 bps with a transcript size of approximately 3.6 Kbs that translates into a 944 aa protein product with a mass of approximately 104 kDa.
- ✓ ORF#93p contains three tandem repeats of the degenerate tetratricopeptide repeat (TPR) domain at its N-terminus that may be involved in chaperonin binding.
- ✓ A monoclonal antibody to ORF#93p has been generated.
- ✓ ORF#93p can bind within PR/chaperonin complexes through PR.
- ✓ ORF#93p when bound within the PR/chaperonin complex, inhibits progesterone binding to PR.
- ✓ Endogenous ORF#93p can be seen in a variety of ovarian cell lines as well as rat brain.

Reportable outcomes:

Results generated over this reporting period are being prepared in manuscript form for submission under the tentative titles:

Graham, D. J.; Abel, M. Greg; Jackson, T. A.; Gordon, D. F.; Wood, W. M.; and Horwitz, K. B. (**2002**) NCRR: a novel chaperonin protein that interacts and mediates the function of the progesterone receptor when bound with antagonist steroid hormone, RU486. (manuscript in preparation).

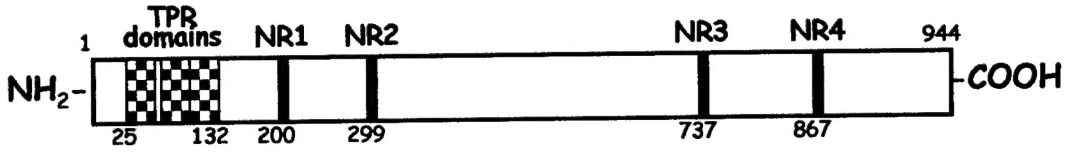
Chaldi, A.; Abel, M. Greg; Graham, J. D.; Horwitz, K. B.; and Toft, D. (**2002**) The scaffolding protein, NCRR, is part of the PR chaperonin complex and influences maturation and hormone binding of the progesterone receptor. (manuscript in preparation).

Graham, D. J.; Abel, M. Greg; Jackson, T. A.; Gordon, D. F.; Wood, W. M.; and Horwitz, K. B. (**2000**) Novel interactors mediating mixed antagonist action on estrogen and progesterone receptors in breast cancer. Keystone Symposia – Nuclear Receptors 2000. (abstract and poster)

Conclusions:

We have identified novel progesterone receptor interacting proteins using an antagonist biased two-hybrid assay. These interacting proteins may be involved in the modification of the transcriptional activity of PR either as direct coregulatory proteins (ie. CBP/p300, N-CoR, and SMRT), as scaffolding proteins involved in the maturation of PR within the chaperonin/heat shock protein complex, and/or as protein folding modifiers that influence the affinity and type of ligand binding to the activated transcription factor. ORF#93p has been identified in a variety of tissues and cell lines and with the generation of a very good monoclonal antibody, the endogenous protein has been identified. A direct influence of ORF#93p on the activity of PR has not been demonstrated but it has been well established that ORF#93p specifically interacts with the C-terminus of PR and that the interaction is influenced by ligand binding. Future work will include the characterization of this hormone dependent interaction and the inclusion of a number of other known PR agonist and antagonist ligands. The nature of the role of ORF#93p in the maturation of PR within the chaperonin complex will be further elucidated using the hsp90 inhibitor geldanamycin. Comparisons between the isoforms of PR (PR-B, PR-A) and ORF#93p binding, influence of ORF#93p on activity of each isoform using cell lines stably expressing either PR-B or PR-A, and ORF#93p recruitment of other auxiliary proteins to the chaperonin complex will be undertaken in the near future. Experiments to being the characterization of the PR interacters also isolated in the original two-hybrid screen (ORF#61 and ORF#127) are being discussed and preliminary data should be generated over the next 6-8 months.

Fig.1 The protein sequence and structural features of ORF#93. TPR (tetratricopeptide repeat) domains are hatched in the cartoon and underlined in the sequence. The four NR (nuclear receptor) boxes are labeled NR1-NR4 and boxed in the sequence. Original fragment, second λ-gt11 library screen fragment, final N-terminal fragment from 5'-RACE.



MTVSGPGTPE PRPATPGASS VEQLRKEGNE LFKCGDYGGA LAAYTQALGL DATPQDQAVL
HRNRAACHLK LEDYDKAETE ASKAIEKDGG DVKALYRRSQ ALEKLGRLDQ AVLDLQRCVS
LEPKNKVFQE ALRNIGGQIQ EKVRYMSSTD AKVEQMFQIL LDPEEKGTEK KQKASQNLVV
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RVVSILGVES QAVSLAACHL LQVMFDALKE GVKKGFRGKE GAIIVDPARE LKVLISNLLD
LLTEVGVSGQ GRDNALTLLI KAVPRKSLKD PNNSLTLWVI DQGLKKILEV GGSLQDPPGE
LAVTANSRMS ASILLSKLFD DLKCDAEREN FHRLCENYIK SWFEGQGLAG KLRAIQTVSC
LLQGPCDAGN RALELSGVME SVIALCASEQ EEEQLVAVEA LIHAAGKAKR ASFITANGVS
LLKDLYKCSE KDSIRIRALV GLCKLGSAGG TDFSMKQFAE GSTLKLAKQC RKWLCNDQID
AGTRRWAVEG LAYLTFDADV KEEFVEDAAA LKALFQLSRL EERSVLFAVA SALVNCTNSY
DYEEPDPKMV ELAKYAKQHV PEQHPKDKPS FVRARVKKLL AAGVVSAMVC MVKTESPVLT
SSCRELLSRV FLALVEEVED RGTVVAQGGG RALIPLALEG TDVGQTKAAQ ALAKLTITSN
PEMTFPGERI YEVVRPLVSL LHLNCSGLQN FEALMALTNL AGISERLRQK ILKEKAVPMI
EGYMFEEHEM IRRAATECMC NLAMSKEVQD LFEAQGNDRL KLLVLYSGED DELLQRAAAG
GLAMLTSMRP TLCSRIPQVT THWLEILQAL LLSSNQELQH RGAVVVLNMV EASREIASTL
MESEMMEILS VLAKGDHSPV TRAAAACLDK AVEYGLIQPN QDGE-944

Fig. 2 Genomic structure of ORF#93. Exons that make up the coding region of ORF#93 are represented as boxes with corresponding exon number. The size (in bps) of the intervening intronic Sequences are below each exon separation. The corresponding basepair numbers of the cDNA coding region of each exon is above. A splice variant found in testis includes the intronic sequence between exons 7 and 8 (circled). The intron sizes between exons 8, 9, and 10 are not known (?) due to the discontinous genomic sequence from chromosome 15 so far published. The STOP codon and poly adenylation signal sequences are labeled in exon 19.

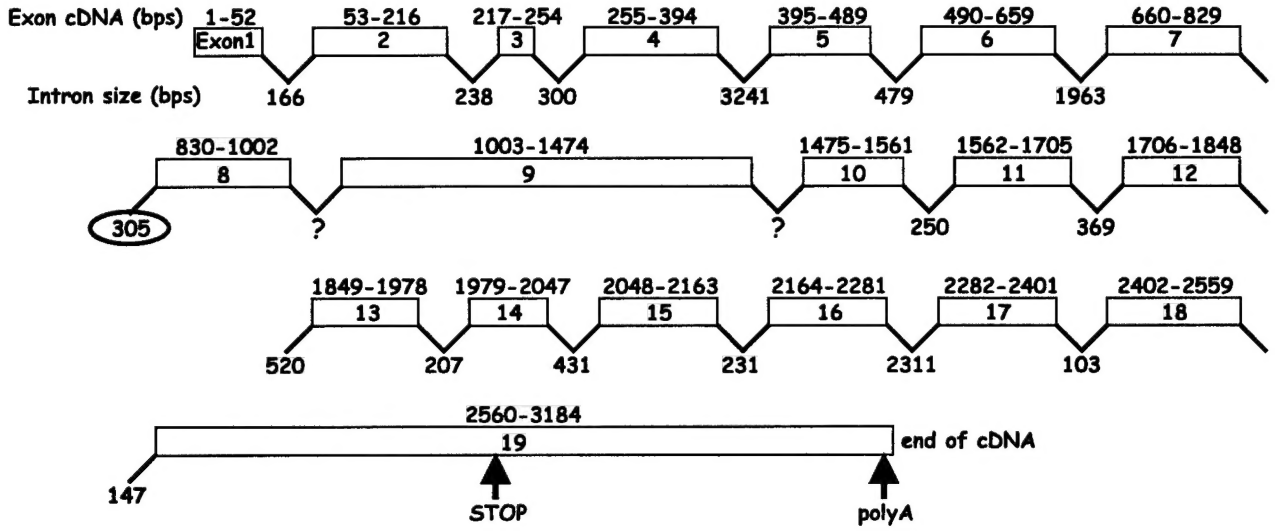
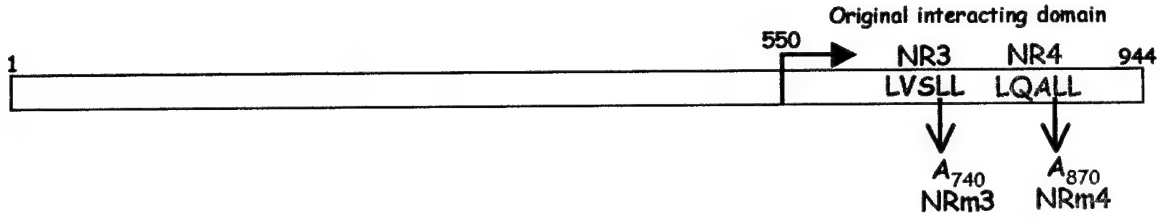


Fig. 3 Mutation of NR3 Abolishes ORF#93 Binding to PR hinge-HBD. The two C-terminal NR boxes of ORF#93, contained in the original 2-hybrid clone (indicated by arrow), were mutated by a single L-->A amino acid substitution in the 4th position of each LXXLL motif. The functional activity of each mutant in yeast cells was assessed by two interaction assays with the hinge-HBD region of PR: one growth based assay (compensation of histidine deficiency) and a color based assay (activation of LacZ). Mutation of NR3 resulted in disruption of protein-protein interactions in both assays. Mutation of NR4 had no effect. The interaction was also demonstrated to be antagonist hormone dependent. The protein-protein interaction was only seen in the presence of RU486.



Yeast 2-hybrid Interaction Assay:

Target Constructs	10^{-6}M RU486	Growth Assay	Color Assay
wt Gal4AD ORF#93 c-term.	−	−	−
wt 550 944	+	+	+
NRm3 A_{740}	+	−	−
NRm4 A_{870}	+	+	+
none	−	−	−
none	+	−	−

Fig. 4 ORF#93 transcript expression. 20ug total RNA from HeLa cells and T-47Dco breast cancer cells were run with molecular size markers on a denaturing agarose gel, and the pGAD93 mRNA was visualized by Northern blotting. The pGAD93 transcript was detected as a single band with an estimated size of 3.6kb.

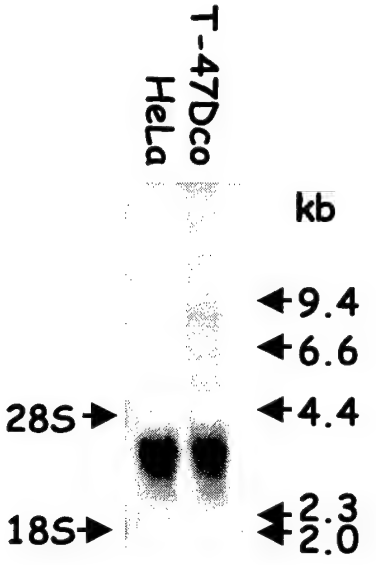


Fig. 5 ORF#93 shares a common chaperonin interacting motif. ORF#93 contains 3 tetratricopeptide repeat domains, a degenerate 34 amino acid repeat implicated in protein-protein interactions and known to bind hsp90. Four LXXLL nuclear receptor interaction motifs are indicated (NR1 - 4). The structural organization of 6 other TPR containing proteins are shown.

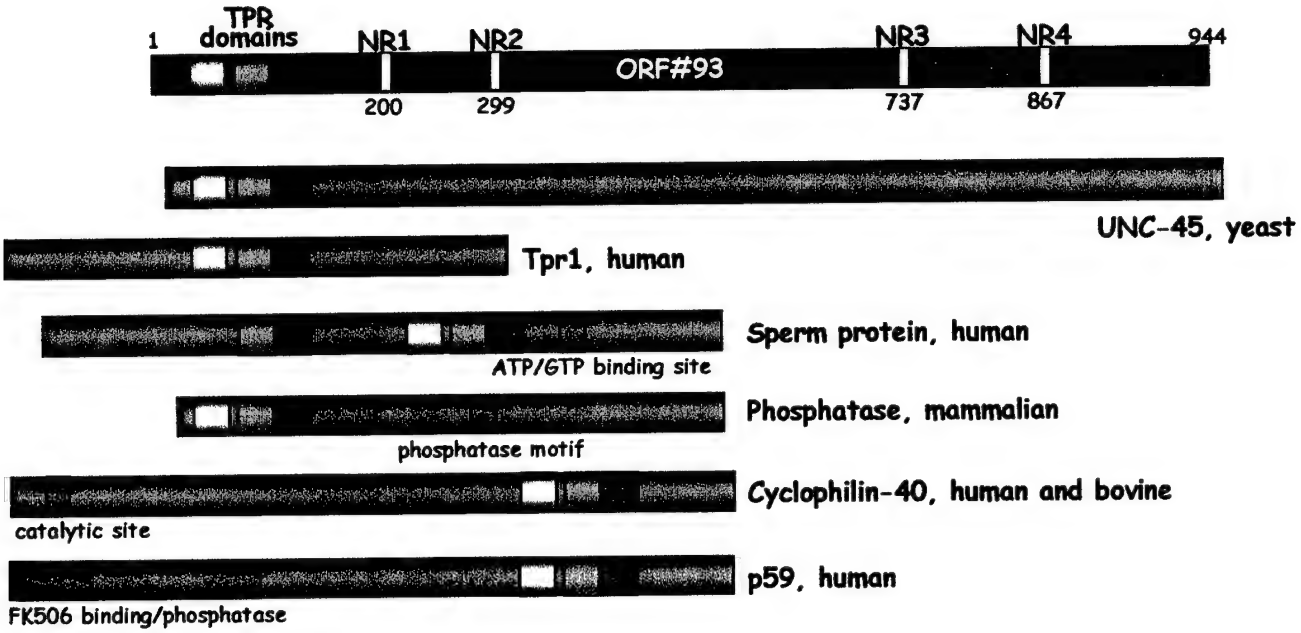


Fig. 6 ORF#93 is recruited in PR reconstitution complexes with purified chaperones. **(A)** Immunoprecipitation where chick PR is absorbed to PR22 antibody resin and then incubated with the 5-protein chaperoning system at 30°C for 30 min. Lane 1: Non-specific resin binding control; lane 2: ORF#93 binding to reconstituted hsp90 chaperonin complex; lanes 3-5: purified proteins run as markers against lane #2. PR22 (chick PR specific antibody resin), 93 (ORF#93 protein), 90 (hsp90 protein), 70 (hsp70 protein), 60 (Hop protein), 40 (hsp40 protein), 23 (p23 protein). **(B)** Western blot with ORF#93 polyclonal antibody.

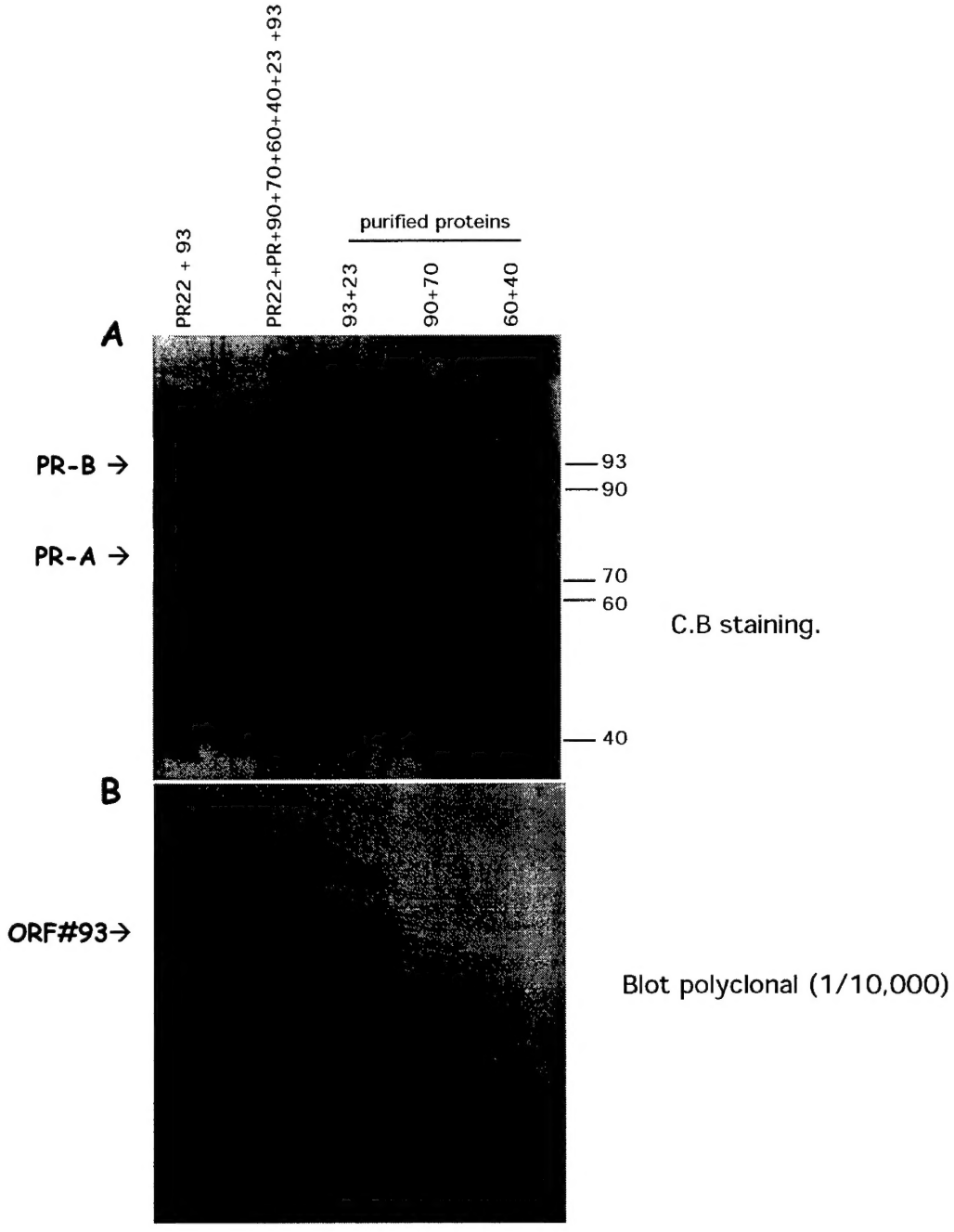


Fig. 7 ORF#93 interacts with PR-A and PR-B in a chaperoning complex and inhibits progesterone binding. **(A)** Lane 1: PR22 resin control showing some non-specific hsp90 binding; lane 2: ORF#93p does not bind to the PR specific antibody resin; lane 3: partial depletion of PR-B from oviduct cytosol with PR6 antibody; lane 4: ORF#93p specifically interacts with PR-A and PR-B after incubation with PR6 antibody; lane 5: normal reconstitution of PR complex; lane 6: when ORF#93p is included in the PR complex, it is evident as the top band - comigrating with PR-B. Binding of ORF#93p to the complex may cause a slight loss of hsp90 binding. **(B)** Tritiated progesterone was used in a hormone binding assay using the various reconstituted complexes shown in lanes 1-6. Binding of ORF#93 to the complex caused a 52% loss in progesterone binding to PR (lanes 5 vs. 6).

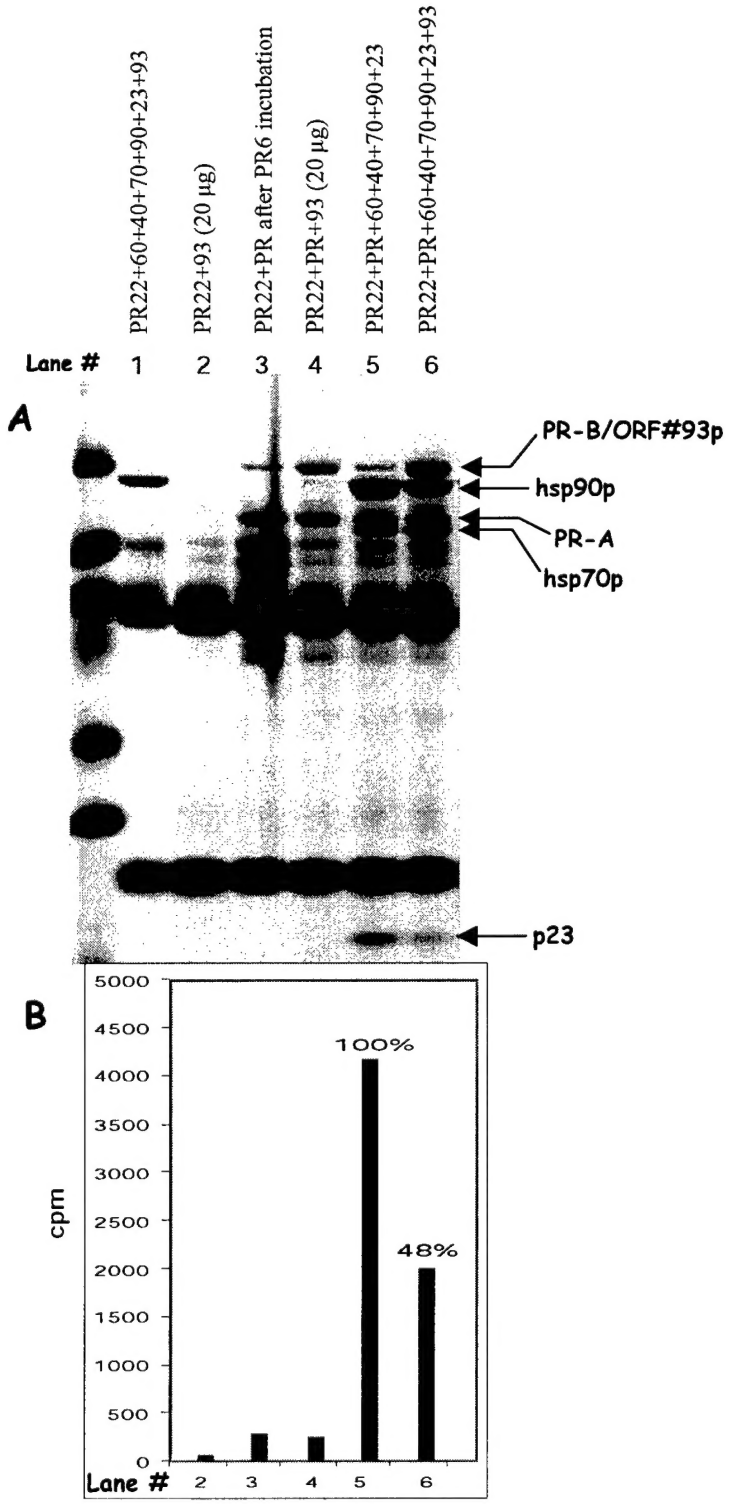


Fig. 8 Western blot of ORF#93 in Rat brain extract. A low level of ORF#93p can be seen in the cytosol (lane 1) which is enriched by IP (lane 2). IP: (20μl resin, 5μl of ORF#93p#2 ascites; blotted with polyclonal antibody 1/5000).

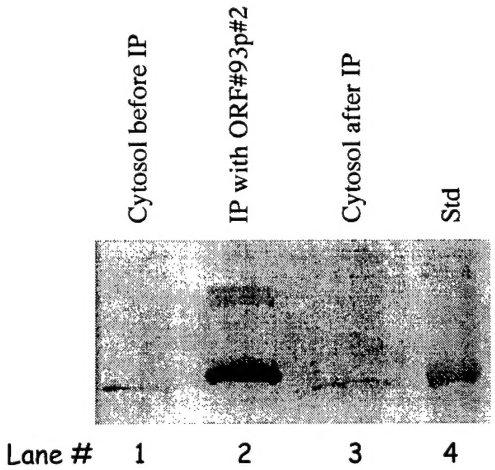
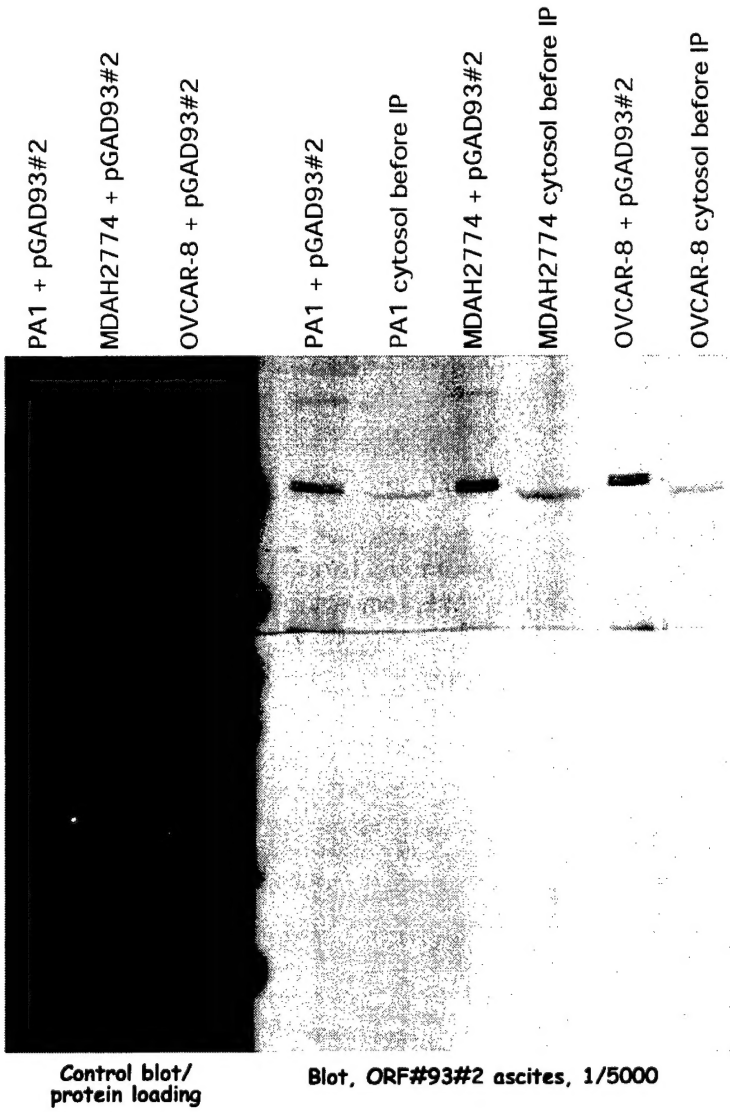


Fig. 9 ORF#93p is expressed in different ovarian cell lines. Using a monoclonal antibody to ORF#93p, we were able to show the expression of ORF#93 in three ovarian cell lines obtained from another lab and enhanced by IP. PA1 (ovary tetratocarcinoma); MDAH2774 (ovarian cancer); OVCAR-8 (ovarian cancer). IP (12.5μl protein G, 5μl ORF#93#2 ascites).



Receptor Interacting Proteins and the Function of Progesterone and Estrogen Receptors in Breast Cancer

J. Dinny Graham, M. Greg Abel, David F. Gordon, William M. Wood and Kathryn B. Horwitz. Division of Endocrinology, University of Colorado Hlth Sc Ctr, B151, 4200 E. 9th Ave, Denver, Colorado 80262, USA.

The nuclear receptors for estrogen and progesterone (ER and PR) are important therapeutic determinants in breast cancer. Tumors expressing both receptors are generally well differentiated, indolent, and likely to respond to treatment with the mixed antiestrogen, tamoxifen. However, responsive tumors inevitably become tamoxifen-resistant and progress, often in the face of continued ER expression. We postulated that this is due to an increase in the partial agonist activity of tamoxifen. To test this hypothesis we have been searching for novel proteins that interact with receptors and modify the activities of mixed antagonists like tamoxifen. Using mixed antagonist-biased interaction screening, we have identified proteins that interact with ER and PR, and regulate transcription. The corepressors N-CoR and SMRT suppress the partial agonist activities of tamoxifen and the antiprogestin RU486, whereas the coactivator L7/SPA enhances this activity, yet has no effect on pure agonists or antagonists. In tamoxifen-resistant tumors removed from patients, we see a trend towards decreased expression of corepressors. In the same screen we identified a cDNA fragment encoding a novel protein, that we have now cloned and sequenced. The 109 kD protein interacts best with unliganded and mixed antagonist-bound PR, and less well with agonist-bound PR. The 944 amino acid protein sequence contains four nuclear receptor interaction LXXLL motifs. Additionally, there are three tetratricopeptide repeat (TPR) motifs in the N-terminus, characteristic of chaperonin/immunophilin binding proteins. Indeed, hsp90 also interacts with the protein strongly in protein interaction experiments. When expressed as a green fluorescent fusion protein, it shows a punctate cytoplasmic localization, which persists in the presence of progestins. We are testing the hypothesis that this protein has a scaffolding function, and plays an integral role in the correct expression and folding of nascent receptors, and perhaps their subcellular localization.